Identification of a new promoter for the response regulator rcsB expression in Salmonella enterica serovar Typhimurium

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Abstract
The RcsCDB (Rcs) phosphorelay system regulates capsule synthesis, flagella production and other cellular activities in several enteric bacteria. This system consists of three proteins: the sensor RcsC, the cognate response regulator RcsB and the histidine-containing phosphotransfer protein RcsD (YojN), which is hypothesized to act as an intermediary in the phosphotransfer from RcsC to RcsB. The rcsC gene is convergently transcribed toward rcsB, which follows rcsD in what appears to be a two-gene operon. Here, it is reported that the overproduction of the rcsB gene represses rcsD transcription, but has a weak effect on its own expression. We demonstrated that the differential rcsD and rcsB expression is due to the activity of two promoters to transcribe the rcsB gene: (1) PrcsDB located upstream of rcsD and (2) PrcsB located within the rcsD coding region. In addition, here it was demonstrated that in Salmonella typhimurium, PrcsB is important to activate the rcsB expression during the stationary growth phase.

Introduction
The RcsCDB system, first identified as a regulator of the capsular polysaccharide synthesis genes in Escherichia coli (Gottesman et al., 1985), consists of the sensor RcsC, the cognate response regulator RcsB and the histidine-containing phosphotransfer RcsD protein. The latter acts as an intermediary in the phosphoryl group transfer from RcsC to RcsB (Takeda et al., 2001; Majdalani & Gottesman, 2005). At present, the Rcs system regulates the transcription of a wide range of genes, including those that encode the master flagellar regulator flhDC operon (Francez-Charlot et al., 2003), the cell division genes ftsA and ftsZ (Carballes et al., 1999), the osmoregulated osmC gene (Davalos-Garcia et al., 2001), the O-antigen chain length determinant wzzB gene (Delgado et al., 2006), the motility and chemotaxis genes (Canó et al., 2002) and also those involved in the Vi antigen synthesis (Virlogeux et al., 1996) in different enteric species.

Although the signal activating the Rcs phosphorelay transduction system remains unidentified, many studies have demonstrated that Rcs activation occurs under certain growth conditions, such as overproduction of DjIA (Clarke et al., 1997; Kelley & Georgopoulos, 1997; Chen et al., 2001), growth at a low temperature, osmotic shock, desiccation (Shiba et al., 2004), growth on a solid surface (Ferrieres & Clarke, 2003), mutation of the tolB gene (Mouslim et al., 2003), the presence of Fe3+ and low Mg2+ in a pmrA mutant (Mouslim & Groisman, 2003), igaA mutation (Canó et al., 2002), mdo mutation (Ebel et al., 1997), as well as the rcsC11 constitutive mutation (Costa & Anton, 2001; Mouslim et al., 2004).

While the rcsB and rcsD genes are situated in what appears to be an operon controlled by a hypothetical promoter located upstream of the rcsD coding region (Blattner et al., 1997; Takeda et al., 2001; Detweiler et al., 2003), the rcsC gene is convergently transcribed to the rcsDB operon by the
PrcsC promoter (Brill et al., 1988; Mizuno, 1997). In the study reported here, we demonstrated that rcsB is transcribed in two different ways: (1) in an rcsD-dependent manner, controlled by the operon promoter, PrcsDB, and (2) in an rcsD-independent manner, under the control of the PrcsB promoter. In addition, the PrcsB stimulates the rcsB expression during the exponential growth phase, while PrcsB does so during stationary growth at lower levels.

Materials and methods

Bacterial strains, molecular techniques and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Recombinant DNA techniques and bacterial growth at 37°C in Luria–Bertani (LB) were performed according to standard protocols (Sambrook et al., 1989). Kanamycin was used at a final concentration of 50 μg mL⁻¹, ampicillin at 50 μg mL⁻¹ and chloramphenicol at 25 μg mL⁻¹.

Introduction of gene fusions and mutations in the chromosomal rcsD, rcsB and rcsC loci

The one-step gene-inactivation method (Datsenko & Wanner, 2000) was used to construct the strains containing a deletion of the rcsD, rcsB or rcsC coding sequence, and PrcsDB and PrcsB promoter regions. The chromosomal ΔrcsD::lacZY, ΔrcsB::lacZY and ΔrcsC::lacZY gene fusion strains were constructed as described (Ellermeier et al., 2002), with the following modifications. The CmR cassette was amplified using pKD3 plasmid DNA as a template and primers 2385 and 2386 for rcsB; 1165 and 1166 for rcsB; and 2803 and 2804 for rcsC (Supporting Information, Table S1). The PCR products were integrated into the chromosome. The junction region of the rcsD, rcsB or rcsC and the CmR cassette was sequenced to confirm the deletion of these genes. After removing the CmR cassette, the lacZY transcriptional fusion plasmid pCE36 was integrated into the FLP recombination target sequence immediately downstream of the genes by FLP-mediated recombination.

β-Galactosidase assays

The β-galactosidase assays were carried out in duplicate, and the activity was determined as described (Miller, 1972). When the bacteria reached an OD₆₀₀ nm = 0.2, ≈ 2 h, 0.35 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the overproduced RcsB regulator from prcsB. After 5 h of IPTG addition, the β-galactosidase activity was determined. All data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the SD.

S1 mapping of rcsD and rcsB promoters

The S1 mapping assay was performed as described (Garcia Vescovi et al., 1996). The RNA was harvested from a wild-type Salmonella typhimurium strain (14028s) growing in LB media, and isolated using the RNA SV total isolation kit (Promega) according to the manufacturer’s specifications. A PCR product was generated with the set of primers 4136/3723 and 4133/3723 for rcsB (Table S1), and chromosomal DNA from wild-type S. typhimurium strain (14028s) was the template and used as the probe. The probe was labeled at the 5' end by phosphorylation with [γ³²P]-ATP using T4 polynucleotide kinase (Gibco BRL) as described (Mouslim et al., 2003).

Table 1: Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028s</td>
<td>Wild-type</td>
<td>Fields et al. (1986)</td>
</tr>
<tr>
<td>EG14873</td>
<td>rcsC11</td>
<td>Mouslim et al. (2004)</td>
</tr>
<tr>
<td>EG14123</td>
<td>ΔrcsC::lacZY</td>
<td>This work</td>
</tr>
<tr>
<td>EG14498</td>
<td>ΔrcsC::Cm</td>
<td>This work</td>
</tr>
<tr>
<td>EG14539</td>
<td>ΔrcsD::lacZY</td>
<td>This work</td>
</tr>
<tr>
<td>EG14931</td>
<td>ΔrcsB</td>
<td>This work</td>
</tr>
<tr>
<td>EG14932</td>
<td>ΔrcsB::lacZY</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prcsB</td>
<td>pUHE2-21 lacR containing the rcsB gene</td>
<td>This work</td>
</tr>
<tr>
<td>prcsBop</td>
<td>pUHE2-21 lacR containing the rcsB gene in opposite direction to the lac promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pMS201</td>
<td>Low-copy vector for cloning promoters, pLtet01, derived from p2S21-luc, gfpmut2, KmR</td>
<td>Beeston &amp; Surette (2002)</td>
</tr>
<tr>
<td>pPracsB</td>
<td>pMS201 containing 122 pb of PrcsB fused to the gfpmut2 gene</td>
<td>This work</td>
</tr>
<tr>
<td>pPracsC</td>
<td>pMS201 containing 131 pb of PrcsC fused to the gfpmut2 gene</td>
<td>This work</td>
</tr>
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</table>

*Gene designations are summarized by Sanderson et al. (1995).
**P_{rcsDB} and P_{rcsB}: gfp fusion construction**

The rcsB cis-regulatory regions, P_{rcsDB} and P_{rcsB}, were amplified from wild-type *S. typhimurium* (14028s) genomic DNA by PCR using the primers 5325 and 5324, and 5327 and 5326, respectively (Table S1). They were cloned into XhoI and BamHI sites upstream of a promoterless gfpmut2 gene (Cormack et al., 1996) in a low-copy number plasmid, pMS201, containing pLtet01 from pZS21-luc (Ronen et al., 2002; Kalir et al., 2005). These derivative plasmids were used to transform the wild-type *S. typhimurium* strain (14028s).

**Determination of promoter activity by the green fluorescent protein (GFP) expression**

Cultures (1 mL) inoculated from single colonies were grown for 8 h in LB medium at 37 °C with shaking at 250 r.p.m. The ON cultures were diluted to OD_{600 nm}=0.003 into LB medium, at a final volume of 150 μL per well in a flat-bottom 96-well plate (Sarstedt). The cultures were grown in a Wallac Victor3 multiwell fluorimeter at 37 °C and set with an automatic shaking repeating protocol; OD_{600 nm} and fluorescence readings were determined as described previously (Ronen et al., 2002; Rosenfeld et al., 2002; Kalir et al., 2005). Measurements were repeated every 6 min, but only the data corresponding to each hour were plotted. Background fluorescence at a given OD_{600 nm} was determined from the fluorescence of cells bearing a promoterless GFP vector at the same OD_{600 nm} as described (Ronen et al., 2002; Rosenfeld et al., 2002; Kalir et al., 2005). All data correspond to mean values of three independent experiments performed in duplicate.

**Results**

**The transcription of rcsD is repressed upon rcsB gene overexpression**

The overexpression of certain response regulator proteins may result in the activation of target genes even in the absence of the cognate sensor or under some inducing condition (Wosten et al., 2000; Oshima et al., 2002; Takaya et al., 2005; Castanie-Cornet et al., 2007). Because the signal and the regulation mechanisms of the RcsCDB system remain unknown, how the rcsB gene overexpression may affect the regulation of the Rcs system was investigated. For

![Fig. 1. rcsB overexpression represses rcsD transcription: (a) Graphic representation of primers (represented by numbers) used to obtain the different deletion fusions in the rcsD, rcsB or rcsC chromosomal genes. The space between brackets represents the region deleted in each mutant, which were replaced by the lacZ gene to obtain the transcriptional fusion. (b) β-Galactosidase activity (Miller units) expressed by strains harboring chromosomal lacZY-transcriptional fusions to the rcsD gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *Salmonella typhimurium* (EG14539) and wild-type (EG14539) carrying the prcsB or prcsBop plasmids, growing in LB medium supplemented with 0.35 mM IPTG as described in Materials and methods. (c) β-Galactosidase activity (Miller units) expressed by strains harboring chromosomal lacZY-transcriptional fusions to the rcsD gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *S. typhimurium* (EG14932), and wild-type (EG14932) carrying prcsB or prcsBop following growth as described in (b). (d) β-Galactosidase activity (Miller units) expressed by strains harboring chromosomal lacZY-transcriptional fusions to the rcsC gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *S. typhimurium* (EG14123), and wild-type (EG14123) carrying prcsB or prcsBop following growth as described in (b). Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the SD.
this, a derivative pUHE21-2lacIq plasmid containing the rcsB gene expressed under the lac promoter (p(rcsB)) and a negative control plasmid with the rcsB gene cloned in the opposite direction of the lac promoter (p(rcsBop)) were constructed (Table 1). A series of isogenic strains, derivatives of the wild-type S. typhimurium strain (14028s) harboring lacZ transcriptional fusions to the rcsD, rcsB or rcsC chromosomal genes, were also generated (Fig. 1a, Table 1). The resulting strains were transformed with the plasmids described above. As shown in Fig. 1b, the levels of rcsD transcription, measured as β-galactosidase activity, in the wild-type strain harboring p(rcsB) were sixfold lower than those observed in the isogenic strain lacking the plasmid or containing p(rcsBop). By contrast, rcsB transcription was reduced only 1.5-fold by rcsB overexpression (Fig. 1c) even when this and the rcsD gene were located in the same operon, and rcsC expression was not affected by this condition (Fig. 1d). These results would indicate that rcsD expression is repressed by high levels of RcsB and suggest that an additional promoter could drive rcsB expression.

**Localization of the rcsDB operon promoter**

It has been postulated that rcsD and rcsB genes are transcribed as an operon, under the control of a putative promoter localized upstream of rcsD (Detweiler et al., 2003). To probe this possibility, we defined this promoter region, localizing the transcription start site of the rcsD gene by S1 nuclease experiments. This assay was carried out using RNA harvested from a wild-type S. typhimurium strain (14028s) growing LB medium and harvested at an OD_{600nm} of 0.6. The S1 protection assay was performed as described in Materials and methods. Lane TC corresponds to the Maxam–Gilbert DNA ladder of the target sequence. The transcription start site and the mRNA sequence are marked by an arrow. (b) DNA sequence corresponding to the 248-bp region upstream of the rcsD ORF. +1 corresponds to the rcsD transcription start site and the bold sequences are the predicted −10 and −35 boxes of the P_{rcsDB} promoter.
Even when it is not usually observed that the *Salmonella* promoters initiate transcription with a C, we had reported the same +1 for the *wzzB* gene (Delgado *et al.*, 2006). In addition, the +1 site allowed us to locate the −35 and −10 boxes at −32 and −10 bp upstream of the *rcsD* transcription start site, respectively (Fig. 2b). This promoter, controlling the expression of *rcsD* and *rcsB* genes, was designated as PrcsDB.

**A new promoter drives *rcsB* expression in addition to PrcsDB**

To further explore *rcsB* gene expression, Northern blot analyses were carried out using RNA harvested from the wild-type strain and the isogenic *rcsC*, *rcsB* and *rcsC11* mutants, after 8 h of growth in LB medium (Fig. 3). The *rcsC11* mutant, harboring a single nucleotide substitution (C to T) at position 477 of the RcsC protein, within the histidine-kinase domain, expresses RcsB-regulated genes even in the absence of a signal (Costa & Anton, 2001; Mouslim *et al.*, 2004). When the complete *rcsB* coding region, amplified by 1106 and 1107 primers (Table S1), was used as a probe, three products were detected: (1) an ≈ 3.5-kb product, which likely corresponds to the *rcsD-rcsB* transcript (Fig. 3, empty arrow); (2) an ≈ 1.28-kb product, which may have originated from degradation of the *rcsD-rcsB* mRNA; and (3) an ≈ 0.67-kb product, corresponding in size to the *rcsB* mRNA transcribed from the 3′ end of the *rcsD* coding region to the predicted *rcsB* p-independent terminator (Fig. 3, filled arrow) (Stout & Gottesman, 1990; Aiso & Ohki, 2003). These mRNAs were present in the wild-type strain as well as in the *rcsC11* and *rcsC* mutants, but were absent in the *rcsB* mutant used as a negative control, indicating that they are *rcsB*-specific products (Fig. 3).

To verify the possibility that *rcsB* would be transcribed from an additional promoter, the 3′ end of the *rcsD* region was examined using the *gen promoter scan* (GPS) program (Zwir *et al.*, 2005). A 424-bp region extending from the 2351 nucleotide of the *rcsD* coding region to the 61 nucleotide of the *rcsB* coding region, including the small intergenic region of 16 bp, was analyzed. The putative promoter was located by this program between the −92 and the −45 nucleotides from the RcsB start codon.

The transcription start site of the putative *rcsB* promoter was detected with the S1 mapping assay using the 424-bp region described above and mRNA from the wild-type strain harvested during the exponential and stationary growth phases. An S1 product was detected under both conditions at the −55 position of the RcsB start codon, demonstrating that the 424-bp region also contains the *rcsB* +1 start site (Fig. 4a, filled arrow). The S1 product increased when the mRNA from the stationary phase was used (Fig. 4a). Taken together, these results support the presence of a new promoter, which was named here *Prcsb* that controls *rcsB* expression in an *rcsD*-independent manner.

**PrcsDB and Prcsb control *rcsB* expression at different growth phases**

In order to explore the relative contribution of the two promoters driving *rcsB* expression, the 122 bp of the *PrcsDB* regulatory region and 131 bp of the 3′ end of the *rcsD* coding region, containing the *Prcsb* promoter, were separately cloned into the reporter plasmid pMS201 (Table 1, Fig. 5a). The activity of each promoter was determined by the expression levels of GFP as described in Materials and methods. In this assay, promoter activity is measured as the rate of GFP production divided by the OD600 nm of culture at each time point (Ronen *et al.*, 2002; Rosenfeld *et al.*, 2002; Kalir *et al.*, 2005). The *PrcsDB*-activated *rcsB* expression after 3 h, during the early exponential growth phase, whereas the *Prcsb* promoted expression after 8 h, when the culture reached the stationary phase (Fig. 5b). Moreover, *Prcsb* displayed a weaker activity than the *PrcsDB* promoter. This assay was also carried out from strains growing at 37 °C in...
50-mL Erlenmeyer flasks at time points of 4, 7 and 10 h, and similar promoter activities as above were observed. These results demonstrate that both promoters are able to support similar promoter activities as above were observed. These

Discussion

It has been reported previously that the expression of the regulon genes of many two-component systems (Soncini et al., 1995; Wosten et al., 2000; Oshima et al., 2002; Takaya et al., 2005) as well as those of the RcsCDB system (Castanie-Cornet et al., 2007) are modulated by high levels of the regulator, even in the absence of the sensor. In this report, for the first time, it is demonstrated that rcsB overexpression significantly inhibits rcsD transcription, but has a weak effect on rcsB expression (Fig. 1), suggesting that the rcsB gene may be transcribed in an rcsD-independent manner. The identification of the PrcsB promoter driving rcsB expression (Figs 4 and 5) as well as a small 0.67-kb transcript corresponding in size to the rcsB mRNA strongly support our notion (Fig. 3). However, Detweiler et al. (2003) suggested that a region of ≈ 500 nucleotides upstream of rcsD was required for the expression of both genes as an operon. Our findings have shown that differential rcsD and rcsB expression is due to the presence of two promoters controlling the rcsB transcription: (1) the PrcsDB suggested previously by Detweiler and colleagues, and (2) the PrcsB described herein. In this work, the –35 and –10 boxes of the PrcsDB promoter were localized at −32 and −10 bp upstream of the rcsD transcription start site, respectively (Fig. 2b).

Furthermore, it was demonstrated that the PrcsB promoter induces rcsB transcription when the bacteria reach the stationary growth phase, while the PrcsDB promoter modulates the operon expression during the exponential growth and is maintained to the later growth phase. In addition, we have shown that even when the PrcsB is able to stimulate the rcsB expression, a weaker activity than the PrcsDB promoter is shown.

The alignment of PrcsDB and PrcsB promoters with equivalent regions from other enterobacteria demonstrated that both are highly conserved (data not shown). In this alignment, strains of E. coli, Klebsiella, Shigella, Enterobacter and

Fig. 4. Molecular analysis of the new rcsB promoter: (a) S1 mapping of rcsB transcripts produced by wild-type Salmonella typhimurium strain (14028s) growing until the exponential (E) or the stationary (S) phase in LB medium. Lane TC corresponds to the Maxam–Gilbert DNA ladder of 170 bp. (b) DNA sequence corresponding to the 163-bp region upstream of the target sequence. The transcription start sites are marked with arrows. (b) DNA sequence corresponding to the 163-bp region upstream of the rcsB start codon (ATG). +1 corresponds to the transcription start site and the bold sequences are the predicted −10 and −35 boxes of the PrcsB promoter.
different Salmonella enterica serovars were included. The localization of Prcsb inside the HPt domain-coding sequence suggests that this promoter could be conserved in all the analyzed strains. The presence of the conserved HPt domain allows the RcsD protein to serve as an intermediate in the phosphotransfer from RcsC to RcsB (Takeda et al., 2001; Majdalani & Gottesman, 2005). We observed that the Prcsb promoter region displays 98% identity between the enterobacteria strains mentioned above (data not shown). This result suggests that the mechanism that controls the RcsB expression could also be conserved.

It was also demonstrated in other two-component regulatory systems that the genes that constitute the system are also transcribed from several promoters. This characteristic has been observed in the phoPQ operon of Salmonella (Sonnini et al., 1995), the phoBR operon of E. coli (Guan et al., 1983), the virA and virG genes of Agrobacterium tumefaciens (Winans et al., 1994) and the bvgAS operon in Bordetella pertussis (Stibitz & Miller, 1994). Perhaps the presence of several promoters in the autoregulation mechanism that control the gene expression constitutes a general feature of these systems. It is important to note here that all of the above-cited systems are under a positive autoregulation mechanism, while the Rcs system is apparently negatively autoregulated. At present, we do not know the pathway by which the RcsB regulator represses rcsD expression; however, ongoing experiments are being directed toward elucidating the mechanism.

In summary, these results demonstrate that two promoters control the expression of the rcsB gene, which may result in an increase in the level of the RcsB protein. The RcsB regulator overproduction represses the Prcsb activity by an unknown mechanism, decreasing the rcsD gene expression. Future experiments are being directed to study the expression regulation of Rcs system component genes.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Primers used in this work.

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